PROVISIONAL PATENT

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PROVISIONAL APPLICATION FOR UNITED STATES LETTERS PATENT

for

ELECTROCHEMICAL DETECTION OF NUCLEIC ACID SEQUENCES

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1.0 Background of the Invention

The government owns rights in the present invention pursuant to grant numbers DAAM01-95-C-0077, DAAM01-96-C-0052 from the *National Science Foundation.*

1.1 Field of the Invention

The present invention relates generally to the fields of sensor technology and molecular diagnostics. More particularly, it concerns the electrochemical detection of nucleic acid segments. The segments are detectable in synthetic and natural environments by coupling electroactively-labeled biological probes to the nucleic acid segment and a colloidal gold electrode.

1.2 Description of Related Art

The determination of a specific DNA or RNA target nucleic acid sequence present in air, food, water, environmental or clinical samples is of great significance in the medical microbiology, food and water safety-testing and environmental monitoring fields. The detection of the presence of a DNA or RNA fragment in a sample can rapidly and unambiguously identify bacterial, viral or parasitic agents of concern. Diagnosis of numerous infectious and inherited human diseases can be done with clinical assays that detect known DNA sequences characteristic of a particular disease (Molecular Diagnostics, 1993; Benn *et al.*, 1987; Lowe, 1986). Unfortunately, few detection methods are suitable for routine diagnostic use either in the clinical laboratory or in the field setting. Many assays are not sufficiently rapid, inexpensive, simple or robust for routine application.

Recent advances in molecular diagnostics have naturally focused on methods of detection at the genetic level. Since the advent of PCR™ technology, the ability to detect point mutations, allelic variation, the presence of minute amounts of a pathogen and identify species or individuals from microscopic samples, to give a few examples, has

been vastly improved. Yet even with the advances of PCR™ technology many limitations still exist which prevent diagnostic assays from being as versatile as desired or needed.

For example, one of the biggest problems with highly sensitive assays, such as PCR™ based assays, is contamination, such as by an extraneous air-borne DNA or by human contact. In general, if a molecular diagnostic assay is highly sensitive and can detect minute quantities of a selected or target nucleic acid segment then the sample to be assayed must be highly purified or at least not contain extraneous nucleic acid fragments which may be at least partially complementary to the target nucleic acid fragment. Otherwise false positive or ambiguous readings can result. Of course, obtaining highly purified samples can be cost ineffective and time and labor intensive. Strong technical expertise and well-equipped diagnostic laboratories are required in most cases. Thus in many instances where a highly sensitive assay is desirable, it is impractical, if not impossible, to perform such assays.

Further, if it is desirable to detect more than one target nucleic acid species in a sample or the sample is highly complex then highly sensitive assays must be refined to detect the desired targets. Although it is not entirely understood, it is well known that many highly sensitive assays suffer from undue interference caused by background sample material. In fact, the time and labor required to refine some assays is so great that the assays are not useful and less sensitive means of analysis must be employed.

Diagnostic assays that are less sensitive are less susceptible to contamination problems and usually require less pure samples but cannot detect minute amounts of target. Thus larger or more concentrated samples must be obtained. In some cases, it is impossible to obtain more sample and in other cases obtaining more sample can be time, labor and cost consuming.

Oligonucleotide-probe, or gene-probe, assays have been developed recently in an attempt to take advantage of the ability to detect DNA or RNA sequences characteristic of

specific bacteria or viruses with high senstivity and replace conventional detection methods. However, these techniques still tend to be labor-intensive and often require significant technical training and expertise. Further highly sensitive gene-probe assays still require specialized equipment and are generally not compatible with field settings. Those that can be used in a field setting usually are limited to determining the presence or absence of a target nucleic acid fragment and cannot meaningfully quantitate it. Thus the utility of gene-probe assasys for environmental montioring and other uses outside of the laboratory setting is limited. Detection assays which can provide a qualitative and quantitative early warning of the presence of pathogens, infectious organisms and parasites harmful to human and environmental health are still needed.

In addition, gene-probes assays often use a label that is either toxic or requires substantial expertise and labor to use. Radiolabeling is one of the most commonly used techniques because of the high sensitivity of radiolabels. But the use of radiolabeled probes is expensive and requires complex, time consuming, sample preparation and analysis and special disposal. Alternatives to radioactivity for labelling probes include chemiluminescence, fluorimetric and colorimetric labels (Kricka, 1993) but each alternative has distinct disadvantages. Colorimetry is relatively insensitive and has limited utility where minute amounts of sample can be obtained. Samples must also be optically transparent. Fluorimetry requires relatively sophisticated equipment and procedures not readily adapted to routine use. Chemiluminesence, although versatile and sensitive when used for Southern blots, northern blots, colony/plaques lift, DNA foot-printing and nucleic acid sequencing, is not inexpensive, and thus is not suitable for routine analysis in the clinical laboratory.

Another limitation to the versatility of oligonucleotide-probe assays is that virtually all current oligonucleotide-probes are designed as heterogeneous assays, *i.e.*, a solid phase support is used to immobilize the target nucleic acid so that free, non-hybridized probe can be removed by washing. Complex procedures and long incubation times (one to several days) are usually required which makes these assays

difficult to incorporate into the simple and rapid formats that are desirable for clinical applications or on-site analysis (Molecular Diagnostics, 1993).

Alternatives to gene probe assay methods of detecting nucleic acid segments have employed electrochemical biosensors that discriminate between immobilized single-stranded and double-stranded DNA (Hashimoto *et al.*, 1994; Millan *et al.*, 1994; Millan and Mikkelsen, 1993). While such biosensors are capable of detecting a known target DNA sequence, they are handicapped by the fact that the electrode must be cleaned between each use. The procedures used to strip away the hybridized target DNA from the electrode surface are not suitable for widespread screening applications, such as clinical diagnostics where labor and expense must be kept minimal and speed is essential, or in settings outside of the laboratory such as the field testing.

For example, coliform counts are currently used to reveal the number of colonies of *E. coli* and related bacteria from samples of water. The culturing step in coliform testing procedure takes considerable time (two to seven days), requires sterile conditions and does not permit the distinction between the types of coliform bacteria present, *e.g.*, those from human fecal contamination, such as *E. coli* and *K. pneumoniae,* and those that are not. Further it is difficult or impossible to cultivate many microorganisms by existing techniques (Chan *et al.,* 1990). Problems with culturing bacteria from coastal waters become even more difficult because bacteria become uncultivable in marine environments (Morton and Oliver, 1994; Munro *et al.*, 1995). Moreover, environmentally induced alterations in cultivability are not unusual, and many microbes are very slow or difficult to culture (McGovern and Oliver, 1995).

Of equal or greater concern is the time required to isolate and identify infectious agents (Schofield, 1992; Panyne and Kroll, 1991). Pathogens in contaminated water and foods obtained from contaminated waters are frequently linked to the incidence and spread of serious diseases. Worldwide, waterborne biological pathogens cause more than 99% of all water-related illness, far surpassing chemical contamination such as lead,

mercury, pesticides and all other forms of contamination. The major problem-causing microbes in water are *Cryptosporidium* and *Gardia*, cholera, hepatitis A, *Salmonella*, typhoid, dysentery as well as various other diarrheal diseases commonly linked to *Escherichia coli*. Some of the pathogens are transmitted by drinking infected water or eating contaminated fish and shellfish; others are spread by bathing, swimming or wading in infected waters; still others are spread by water-based insects and snails (Lee, 1985).

Clearly, there is a need for improved detection of nucleic acid segments that may be identified with specific pathogens. There is also a need for more rapid, less labor intensive and cost effective clinical assays for the detection and identification of diseases and disorders affecting mankind as well as field portable assays and kits which can be used to monitor the sources of such infections. Unfortunately, few assays are currently available for routine monitoring and/or diagnostic use because of the expense, complexity and/or physical limitations which prevent their use outside of a well-equipped laboratory. As discussed, the few assays that do exist have limited applications and do not meet the diverse needs of clinical diagnostics and field testing.

2.0 Summary of the Invention

The present invention seeks to address these and other deficiencies inherent in the prior art by providing a simple and sensitive electrochemical method of detecting virtually any tyupe of nucleic acid segment, provided the target nucleic acid has been identified. Thus the present invention in a broad aspect resides in an electrochemical system for detecting specific nucleic acid segments, including DNA and RNA. In a more specific aspect the invention utilizes oligonucleotide segments specific for identified gene sequences. The invention has particular application for detecting identified nucleic acid sequences in complex mixtures. A further aspect of the invention is a utility for assaying virtually any species, whether microbial or higher life forms, so long as an identifiable gene segment can be determined. Diagnostic assays, such as for aberrant chromosomal variations, cancers and genetic abnormalities are also part of the invention to the extent

that targeted nucleic acid sequences can be selectively probed employing the disclosed methods.

The invention may be viewed in several different contexts: it may be viewed as a method involving the capture of an electrochemically labeled nucleic acid on a colloidal gold electrode; it may be viewed as an electrode for use in the electrochemical identification of gene segment analytes; it may also be viewed as an electrochemical system for assaying for a nucleic acid analyte as in identification of a bacterial species, for example, or in a method of diagnostic assay.

The present invention couples the simplicity and sensitivity of electrochemical techniques with gene-probe hybridization assays to detect specific types of DNA or RNA through direct analysis.

The invention in one broad embodiment comprises hybridizing nucleic acid sequences characteristic of a target species, whether prokaryote, eukaryote or other species which have a characteristic nucleic acid sequence, with a selected biological probe capable of specifically hybridizing to a "target" nucleic acid sequence. Thus, the biological probe hybridizes with a target sequence. It is particularly advantageous in that optically transparent samples are not required nor is the purification or isolation of the target nucleic acid sequences from the sample required.

Target nucleic acid sequences may vary widely. Especially attractive target-nucleic acid sequences are those found in various microbes where it is desirable to detect the presence of such microbes in food, water or air. In such cases, a sample comprising the target-nucleic acid segments may be subjected to lytic conditions prior to analysis in order to increase the ready access of the target nucleic acid segments for hybridization with the biological probes. The selected nucleic acid segment may be detected in a complex mixture such as whole blood, lysed bacterial samples, culture media, ground meat samples, the surface of a meat sample, biofilms, soil, fish slime,

water from fish tanks, marine sediment, marine or freshwater samples, for example, and can be used in the diagnosis of infectious diseases, in environmental bioremediation, in the determination of genetic disorders, in genetic epidemiology and research.

In many cases, the invention eliminates the need for using PCR™ based technology, cell culture or other methods of selectively amplifying a target nucleic acid fragment.

The invention provides a method of electrochemically detecting a target nucleic acid fragment. A DNA or RNA sequence is "captured" or hybridized at a working or "test' electrode surface by a gene-probe. The captured target is then electrochemically detected by applying an amperometric potential across a working electrode and a reference electrode such that a current is generated which then flows between the working electrode and one other electrode. Measurement of a current indicates the presence of the target nucleic acid segment.

In one embodiment, only two electrodes are present, in which case the current flows between the working and reference electrodes. Alternatively, three electrodes are present; a working, reference and counter electrode, in which case the current flows between the working and the counter electrodes.

One, two or more gene-probes may be employed. In such instances where two or more probes are used, there should be no sequence overlap between the two or more selected target areas of the nucleic acid sequence or sequences. The target nucleic acid sequences may be adjacent to each other or widely separated from each other.

In a typical 2-probe assay method, each biological probe comprises an oligonucleotide segment coupled or covalently bound with another molecule such that one of the biological probes (termed the capture probe) is capable of hybridizing, bonding, binding or otherwise coupling to a working electrode of an electrochemical cell

and the other probe (termed the detector probe) is capable of hybridizing, bonding, binding or otherwise coupling to an electroactive label or electroactive reporter group. When both the capture probe and detector probe are hybridized to their respective target nucleic acid sequences and respectively hybridized, bonded, bound or otherwise coupled to a working electrode and electroactive label, a complex is formed that functions as an analyte in an electrochemical cell to produce a catalytic current when an amperometric potential is applied.

Alternatively, in a one probe assay method, a biological probe that comprises an oligonucleotide segment is hybridized to a target nucleic acid segment and coupled to a working electrode such that when an amperometric potential is applied across the working electrode and a reference electrode then a current is generated which flows between the working electrode and another electrode. The latter electrode may be the same reference electrode or preferably a third electrode, designated herein as a counter electrode.

An important aspect of the present invention is the electrode biosensor and the electroactive label coupling which promotes a strong, quantifiable catalytic current only when a "bridge" is made by the hybridization of the biological probe with the complementary target nucleic acid sequence. At the molecular level, this "bridge" puts the electroactive label in close proximity to the electrode, such that a current is generated when a amperometric potential is applied across the working and reference electrodes.

The electrochemical cell comprises two or more electrodes. One of the cell electrodes serves as a reference electrode. The invention includes the use of disposable working electrodes which have ultramicro-arrays of colloidal gold particles on the surface. There is one working electrode present for each different probe-target complex that is to be detected. An amperometric, potentiometric, or other suitable electrometric device serves to measure current generated between the working electrode and another, either reference or counter, electrode.

The potential imposed on an electrochemical cell of the invention can be of a constant value, thereby generating a generally constant or steady-state current. A preferred potential is a "pulsed" potential, *i.e.* a series of brief, intermittent potentials of generally constant amplitude that are applied or pulsed through the electrochemical cell. The pulses may also alternate between two voltage levels on each side of a common voltage level. An especially preferred pulsed potential is an intermittent one in which the potential is simply disconnected from the electrochemical cell at regular intervals.

The preferred current detection range for the quantitation of a target nucleic acid segment is between about 0 μA and about 10 μA. Qualitative detection of a selected nucleic acid fragment can be between about 0.5 μA and about 20 μA, with the preferred range being between about 0 μA and about 10 μA.

A bonding agent preferably bonds the capture probe to the colloidal gold electrode. A bonding agent may be a protein, oligonucleotide or other molecule that is capable of bonding to both the working electrode and the capture probe. A preferred bonding is achieved by incorporating a protein, such as avidin or streptavidin, or an oligonucleotide to the colloidal gold electrode and also incorporating a molecule, such as biotin, or another oligonucleotide, complementary to the oligonucleotide already attached to the colloidal gold electrode, which is capable of binding, bonding, hybridizing or otherwise coupling with both the bonding agent and the oligonucleotide of the capture probe.

In certain cases, target nucleic acids labeled or tagged with an electroactive label may bond sufficiently to a colloidal gold electrode to obviate the need for a special capture probe. In most instances, it will be desirable to couple oligonucleotides to more than one sequence in a target nucleic acid as this may provide improved signal strength and accuracy in identifying target nucleic acid.

An electroactive label can be added to the target nucleic acid segment, or to any biological probe or it can be inherent in the target.

In certain embodiments an electron mediator and a substrate can be present in the electrochemical cell. An exemplary electron transfer mediator is ferrocene monocarboxylic acid (Fc). An exemplary substrate is peroxide. The substrate and electron transfer mediator are added to the aqueous medium after hybridization of the gene-probes with target nucleic acid sequences and, if necessary as separate steps, allowing the hybridization product to couple with an electroactive label and the electrode biosensor.

Detector probes of the present invention may be coupled directly or indirectly with electroactive reporter groups such as horseradish peroxidase (HRP) so that a strong catalytic current is produced from hybridized HRP-labeled probes, for example, captured on the electrode surface. The indirect coupling of the detector probe to the electroactive reporter group is achieved through the attachment of another molecule to the oligonucleoitde sequence of the detector probe. This molecule may be, but is not limited to, biotin, fluorescein, digoxigenin, a thiol group or another oligonucleotide sequence.

A variety of electroactive labels can be incorporated in the disclosed invention. A preferred electroactive reporter group is horseradish peroxidase (HRP). Other electroactive reporter groups that can be used include, but are not limited to, microperoxidase, alkaline phosphatase and thiol groups inherent in the target nucleic acid segment.

More than one target DNA or RNA species may be detected from a sample, that is, more than one target-nucleic acid sequence may be hybridized to a distinct oligonucleotide-probe or set of probes. For example, target-nucleic acid sequences from both *E. coli* and *Salmonella* may be hybridized to different gene-probes such that two different hybridized probe-target complexes are formed: one which comprises nucleic

acid sequence from *E. coli* and one which comprises nucleic acid sequence from *Salmonella*. Each hybridized target nucleic acid sequence is capable of coupling, binding, bonding or hybridizing to a working electrode that is specific to that hybridized target nucleic acid sequence.

In another embodiment, the disclosed invention can be coupled to polymerase chain reaction (PCR™) amplifications such that the DNA from a pathogen is amplified or the RNA from a pathogen is reverse transcribed (RT) and then subjected to PCR™ and the amplified product is detected electrochemically. The amplification and detection of target nucleic acids can also be automated by combining polymerase chain reaction, PCR™, and electrochemical detection into a single unit.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

3.0 Brief Description of the Drawings

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. DC Pulse Amperometric Detection.

FIG. 2. Pulsed Electrochemical Detection. The potential waveform shown here is used in HPLC detection of carbohydrates. Current is measured at the end of the detection pulse which is part of a three pulse sequence repeated every 60 milliseconds.

FIG. 3. Differential Pulse Detection. In this example, a potential waveform comprised of 45 millisecond base pulses and 5 millisecond detection pulses is used at 20 Hz frequency, with currents measured at the end of each pulse.

FIG. 4. Intermittent Pulse Detection. The waveform used here consists of 5 millisecond pulses of -100 mV potential, separated by 45 millisecond intervals when the sensors is disconnected from the electronics of the Monitor. This particular waveform was used successfully to increase current signals detected in the *E.coli* assay of the claimed invention.

FIG. 5. Schematic of the screen-printed circuits and the electrodes on the plastic test strip.

FIG. 6. Effect of additional fluorescein moieties to the detector probe on the electrochemical signal measured.

4.0 Description of Illustrative Embodiments

The present invention provides methods and compositions for the selective, rapid, and sensitive electrochemical detection of nucleic acids such as are found in microbes, including bacteria, viruses and parasites that may be present in food, air, fresh and/or marine waters; other environmental samples; or biological samples, such as tumor cells and other cells exhibiting abnormalities. Of ourse any nucleic acid may be detected so long as its sequence is known or sufficiently identified to allow selection of appropriate hybridization probes. The methods are applicable to the analysis of samples in a clinical, research or outdoor, *i.e.* field, setting and are also useful for monitoring in genetic epidemiology and environmental remediation.

The invention provides an electrochemical detection system that utilizes electroactive biological probes and electrode biosensors for the specific detection of

target-DNA at the electrode surface. Homogeneous electrochemical assays are provided, *i.e.* assays that utilize a single-tube format such that a solid phase is not required to immobilize the target-DNA or the hybridized target-DNA/gene-probe in order for detection to occur. The present invention shows sensitivity and selectivity equal or better than conventional methods in a more rapid, less expensive and simpler-to-use format.

Distinct advantages of electrochemical detection combined with gene-probe methodology are readily apparent. A detectable signal can be generated in minutes as opposed to hours, as with many colorimetric assays, or even days, as with many radioassays. Harmful and increasingly difficult to dispose of materials, such a radioisotopes or mutagenic colorimetric labels, are not required. Quantification of the signal is easily accomplished and a pathogen can be successfully detected with as little as a picogram to femptogram (10^{-12} to 10^{-15}) of target DNA. Coliform bacteria can be detected at ten-fold fewer pathogens than at the regulatory level of 200 cells per 100 ml of sample, *i. e.*, *Escherichia coli* can be detected in a sample with as few as 200,000 *E. coli* cells/ml of aqueous sample.

Examples of pathogens that can be detected using the invention include, but are not limited to, bacteria such as *Salmonella, Escherichia coli, Klebsiella pneumoniae, Bacillus, Shigella, Campylobacter, Helicobacter, Vibrio,* parasites such as *Giardia, Naegleria* and *Acanthamoeba* and such as viruses *Hepatitis* and poliomyelitis.

By using specific sequences of DNA or RNA that are characteristic of target microbes, pathogens can be unambiguously identified, regardless of their cultivable states, by direct analysis of contaminated food or water samples. Thus more definitive data are provided regarding food and water quality and the time-consuming culturing step associated with coliform counts is eliminated. In addition, distinctions can be made between different coliform bacteria, *e.g.* pathogenic *v.* nonpathogenic bacteria.

The invention is not only useful for the detection of food pathogens and water-borne microbes, but also may be employed to detect genetic variation associated with different disorders or diseases. Examples of diseases that can be detected by the present invention include cystic fibrosis, muscular dystrophy, sickle cell anemia, phenylketonuria, thalassemia, hemophilia, a_1-antitrypsin deficiency, disorders of lipoprotein metabolism and inherited forms of breast cancer. In addition, quantitative analysis of human genes is also desirable for analysis of amplified oncogenes (Altitalo, 1987) and in the determination of gene expression levels in tumors (Slamon *et al.*, 1989).

The present invention improves upon current gene-probe assays by requiring fewer steps to perform, detecting specific targets at lower concentrations and needing less time to complete. A particular advantage of the present invention is that it can be used outside of a well-equipped laboratory setting. Complex instrumentation is not required because the gene-probes and electrodes are employed with an inexpensive, hand-held meter or fieldable monitor. These electrochemical assays can be automated in a number of ways using relatively inexpensive equipment and procedures that are generally more robust and less complex for the operator to perform than comparable homogenous immunoassays.

The disclosed invention shows that gene-probes may be effectively coupled to colloidal gold electrodes such that a target-DNA is detected with high sensitivity. Examples herein demonstrate the increased sensitivity achieved by the disclosed electrochemical gene-probe methods as compared to conventional methods of detecting coliform bacteria in samples collected from marine/freshwater environments or food extracts. The electrochemical detection system of the present invention provides an improved means of monitoring human and environmental health through food and water-safety assays.

The quantifiable electrochemical signal results from electroactive groups being held in close proximity to the electrode surface by the immobilized probe-target. Free

electroactively labelled probe in solution that is not immobilized on the electrode surface does not couple efficiently enough to the electrode to produce an electrode response (O'Daly *et al.*, 1992); thus no separation of free probe from the hybridized material is needed.

The electrochemical detection system of the present invention takes advantage of gene probes. These gene-probes can be described as biological probes, nucleic acid-probes, DNA-probes, or oligonucleotide-probes. The present invention comprises a gene-probe, the "capture probe", and may also comprise a second probe, the "detector probe". Optionally, three, four, five or more probes may be used.

As defined herein, each gene-probe comprises at least an oligonucleotide sequence, which is complementary to a contiguous nucleic acid segment of an identified, target pathogen such that the oligonucleotide sequence specifically hybridizes to the nucleic acid sequence of the pathogen under conditions of high stringency. Oligonucleotide sequences of 18 to 50 nucleotides are preferred; however, shorter or longer sequences may in certain instances be employed; *e.g.*, 15, 16, 17, *etc.* or even 51, 52, *etc.* as well as any number of 19, 20, 21, *etc.* up to 50 or so nucleotides.

As used herein the term "complement" is used to define the strand of nucleic acid which will hybridize to the first nucleic acid sequence to form a double stranded molecule under stringent conditions. Stringent conditions are those that allow hybridization between two nucleic acid sequences with a high degree of homology, but precludes hybridization of random sequences. For example, hybridization at low temperature and/or high ionic strength is termed low stringency and hybridization at high temperature and/or low ionic strength is termed high stringency. The temperature and ionic strength of a desired stringency are understood to be applicable to particular probe lengths, to the length and base content of the sequences and to the presence of formamide in the hybridization mixture.

In certain embodiments where at least two probes are used, either the capture probe or the detector probe may hybridize to any one of a generic group of related targets of interest. In such cases, it is generally preferred that the other probe be specific to a particular target species. Alternatively, one may choose to use both a capture probe and a detector probe that hybridize to any one of a group of related targets if the objective is to measure the occurrence of a particular generic group. For example, a detector probe can hybridize to 16s rDNA of both *E. coli* and *Salmonella*, and a first capture probe can also hybridize to 16s rDNA of both *E. coli* and *Salmonella*. An electrochemical signal generated by using these two probes would indicate that either or both *E. coli* and *Salmonella* were present in the sample. Alternatively, a second capture probe which only hybridizes to nucleic acid segments of *E. coli* could be used in place of the first capture probe. An electrochemical signal generated by using the detector probe and the second capture probe would only indicate the presence of *E. coli* in the sample. *Salmonella* nucleic acid segments that are hybridized to the detector probe would not be detected since these the *Salmonella* nucleic acid segments are not coupled to the electrode.

In certain embodiments, the oligonucleotide sequence of a gene-probe is bound, bonded, conjugated or otherwise coupled with either a protein, such as biotin, or an antibody; or another molecule, such as fluorescein (FL) or dioxigenen (DIG), that is able to bond with an electroactive reporter group; or is bonded directly to a biosensor electrode; or is able to hybridize to another molecule, such as an oligonucleotide or protein, such as an avidin, which is bound to a biosensor electrode.

Herein in certain cases, the capture probe includes a component, such as biotin or anti-fluorescein antibody, that is reactive with a component of the colloidal gold electrode, such as avidin, streptavidin, protein G or protein A so that the capture probe becomes bound to that component of the electrode or is itself bonded, bound, conjugated or otherwise coupled directly to the colloidal gold electrode.

In some cases, the detector probe includes at least one molecule, such as fluorescein (FL) or digoxigen (DIG), that can be coupled, conjugated, bound or otherwise bonded to an electroactive label, such as horseradish peroxidase (HRP), or is directly coupled, conjugated, bound or otherwise bonded to an electroactive reporter group.

In other embodiments, the protein conjugated to the oligonucleotide of the capture probe is biotin, at least one molecule of is FL or DIG is conjugated to the detector probe and the electroactive reporter group is HRP. Alternatively, either gene-probe or both may recognize an antibody such as HRP-anti-fluorescein antibody.

An especially effective component for use in the invention is an electrochemical monitor. This apparatus is sufficiently compact to be hand held and operates on samples which are mounted on disposable test strips. Each test strip is inserted through a small opening into a box-like structure. A sample well on the test strip contains a sample such as a target nucleic acid segment/oligonucleotide-probe hybrid of the invention. The sample well is located over a colloidal gold electrode which is screen-printed on the test strip together with a reference (preferably silver) electrode and a counter (preferably carbon) electrode.

In certain applications, a material (*e.g.*, streptavidin) conjugated with the colloidal gold bonds the colloidal gold electrode to the target nucleic acid/oligonucleotide/redox reporter. An oxidizing agent (*e.g.*, hydrogen peroxide) added to the sample generates a redox reaction which, in turn, generates an electrical current indicative of the target nucleic acid in the presence of an amperometric potential. The signal is measured by an amperometric or other suitable electrometric instrument.

In general, an analysis of the type disclosed above requires no more than about 10 or 15 minutes to perform. The signals generated are typically proportional to the amount (concentration) of target nucleic acid present in the sample.

The present invention provides methods for the quantitative detection of microbes in a sample. The source of the sample may be food, water or a biological or clinical sample which can be treated such that material suspected of containing nucleic acid segments from the microbes can be freed into an aqueous medium and subjected to lytic conditions designed to release the nucleic acid segments characteristic of the microbes from the material into the aqueous medium such that the capture and detector probes hybridize with their respective target regions of the nucleic acid segments. The aqueous medium is such that an electrochemical signal is generated by the hybridized probes/nucleic acid segments and detected by an electrode biosensor. In certain cases, an electron mediator and an electroactive reporter group are used to generate an electrochemical signal.

The present invention also provides an electrochemical detection system which uses only a capture probe. In this instance, the electrochemical signal is generated when the target-probe hybrid is immobilized on the working electrode, an amperometric potential is applied across the reference and working electrodes and an electrochemical current flows between the working electrode and another electrode in the presence of an electroactive group that is either inherent in the target nucleic acid segment, *e.g.* a thiol group, or is chemically added to the target.

The invention also provides easy-to-use kits which contain monitors, reagents and procedures that can be utilized in a clinical or research setting or adapted for either the field laboratory or on-site use. These kits can be widely employed in less technologically developed areas or countries which do not have well-equipped laboratories and at remote sites far from well-equipped laboratory facilities. The invention thus is useful in monitoring for the presence of microbes which cause waterborne diseases at both water treatment plants and at untreated water supplies such as a river or lake.

4.1 Use of Gene-Probes in Electrochemical Detection

The present invention couples gene-probe technology with sensitive electrochemical detection. The basic system for electrochemical detection is one of coupled reactions, such as the HRP electrode assays previously developed (US Patents 5,217,594; 5,225,064; 5,334,296; 5,368,707; 5,391,272 all incorporated herein by reference, and also described in O'Daly *et al.*, 1992; Zhao *et al.*, 1992; Henkens *et al.*, 1987; 1991; 1992a; 1992b; Stonehuerner *et al.*, 1992; Crumbliss *et al.*, 1990; 1992; 1993). An electrode is bonded to a capture probe which, in turn, binds a target nucleic acid segment with high specificity.

In certain cases, the electrode is further prepared by binding to the immobilized capture probe/target-nucleic acid segment a detector probe which is conjugated to an electroactive reporter group, for example horseradish peroxidase (HRP), which can transfer electrons. When suitably combined with a reference and working electrode, the capture probe/target-nucleic acid segment/detector probe/electroactive reporter group complex, in the presence of a substrate for the electroactive reporter group, such as peroxide, and an electron mediator, causes a measurable and quantifiable electrical current.

4.2 Homogeneous Hybridization and Electrochemical Detection

The assay chemistry can be conveniently divided into four general steps: (1) sample treatment/cell lysis, (2) hybridization, (3) hybrid capture, and (4) detection.

4.2.1 Sample treatment/cell lysis

Samples are collected and concentrated or lysed, as required. Sample concentration is accomplished by a membrane filter technique accepted by the Environmental Protection Agency (EPA) for microbiological testing of potable water (11th edition, Standard Methods for the Examination of Water and Wastewater and incorporated herein by reference). In traditional assays the filter is placed in a petri dish

with nutrient media for several days for growth of bacterial colonies. These culturing steps are not required by the present invention.

Appropriate adjustment of pH, treatment time, lytic conditions and sample modifying reagents may be altered in order to optimize reaction conditions. Such modification techniques are well known to those of skill in the art and are described in Maniatis *et al.*, 1989 and Ausubel *et al.*, 1989, incorporated herein by reference. Although culturing steps are generally undesirable, procedures that include short term culturing prior to measurement are desirable in some cases. Such assays by the disclosed method would still have shortened analysis time compared to conventional assays because it is unnecessary to grow organisms to the point of visible colonies.

4.2.2 Hybridization

Hybridization of the target DNA and oligonucleotide probes is generally carried out in an aqueous solution which contains an excess amount of probes, or alternatively contains a detector probe if the capture probe is immobilized to the colloidal gold electrode biosensor and two probes are used. In either case, hybridization proceeds rapidly because both the target DNA and probes are in a homogeneous hybridization system rather than a heterogeneous hybridization system.

In a homogeneous hybridization system, hybridization of probes and target DNA occurs in solution and the resultant hybrid does not need to be transferred to a solid support surface, such as a nitrocellulose filter, in order for detection to take place. In conventional filter hybridization methods such as Southern blot hybridization (Southern, 1975), the target DNA or RNA is immobilized on a filter membrane and then allowed to react with the probe molecule. After the hybridization reaction is complete, excess probe molecules are removed by washing the filter and the labeled hybrids are detected by autoradiography or by a non-radioactive detection method.

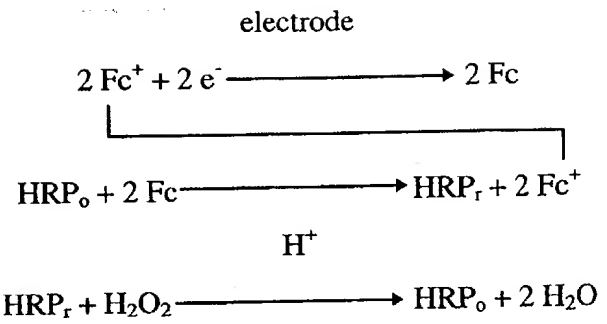
In these conventional methods the rate of hybridization is very slow because the target is present in low concentration and immobilized on a surface (Flavall *et al.*, 1974). Overnight or longer incubations and autoradiographic exposures are sometimes required to obtain good sensitivity. Protocols of the claimed invention are faster because they are designed to increase hybridization rates by carrying out reactions in solution and capturing the DNA hybrids afterwards (Albretsen *et al.*, 1990). Thus the amount of time, labor, expense and technical expertise required is reduced.

4.2.3 Hybrid capture

The present invention allows identification of specific nucleic sequences in crude samples. In certain cases, it is desirable to use two different probes, *i.e.* a capture and a detector probe. This hybridization technique can be more specific than single probe hybridization because two hybridization events must occur in order to generate a signal. The gene-probe electrodes are coated with molecules, such as streptavidin, avidin or even the capture probe, designed to capture target-DNA/detector probe hybrids.

In embodiments which include a detector probe, a strong catalytic current is produced when target DNA-probe hybrids that are immobilized on the electrode surface and labeled with an electrochemically detectable label, such as HRP, are exposed to an amperometric potential in the presence of a substrate, *e.g.* peroxide. The result of catalysis by the HRP label is a flow of current between the working electrode and another electrode, either the reference electrode in a two electrode system or the counter electrode in a three electrode system. The current is measured by a monitoring device.

Equations 1-3 illustrate the electrochemical detection of HRP bound at an electrode. An electron transfer mediator is used in this system. A preferred mediator is ferrocene monocarboxylic acid (Fc).



At the molecular level, capture at the colloidal gold biosensor puts the HRP in close proximity to the electrode, where it generates a current in the presence of peroxide. The peroxide is added as a final step, equation 3, after allowing the probe and target sequences to hybridize. The electrochemical detection assay exhibits rapid response with a good signal to noise ratio.

Another electron transfer mediators that can be used in the claimed invention is 3,3',5,5'-tetramethylbenzidine.

The hybridization solutions can comprise buffers of relatively low stringency, as determined by previous hybridization studies.

4.2.4 Detection

The sample containing hybridized target DNA/detector probe, or hybridized capture probe/target-DNA/detector probe or hybridized capture/target DNA, is applied to a "test strip" which contains the electrode biosensor and comprises at least a working electrode and a reference electrode. A third, counter, electrode is also present if quantitation of microbial concentration is desired. Hybrids are captured at the electrode surface by the reaction of the capture probe with the electrode biosensor or by the hybridization of the target-DNA/detector probe hybrid with the capture probe that is already bound to the biosensor.

Although an excess of capture probe is present, either bound directly to the biosensor or in the aqueous hybridization solution, no catalytic current or electrochemical signal is generated by these non-hybridized capture probes because these capture probes are not also attached to an electroactive reporter. In one preferred embodiment, the capture probe is biotinylated and the electrode biosensor is coated with colloidal gold and avidin, preferably a synthetic avidin.

4.3 Types of Electochemical Detection

4.3.1 Conventional DC Amperometric Detection

Most electrochemical systems employing electrochemical sensors such as those for blood glucose, oxygen, or hydrogen peroxide, as well as immunosensors and gene-probe sensors, involve measuring currents using DC Amperometric Detection (FIG. 1), which is also referred to as Amperometric Detection. In this method a constant potential is continuously applied to the working electrode and, after allowing sufficient time (usually, several seconds), a "steady state" current is measured. The current results from an electrochemical process induced by the applied potential, in which the analyte electrochemically either reacts at the working electrode or is involved in a reaction cycle with some other species reacting electrochemically. In order to have analytical utility, the measured current signal has to have predictable and stable correlation to the concentration of the analyte. The same, of course, has to be true for any other detection method. The major drawback of the DC Amperometric Detection is that the properties of the electrode interface change in time as a result of continuously applied potential. The change, usually called electrode fouling, may be due to adsorption of sample components (*e.g.*, proteins, lipids) on the surface of the sensor which changes the current signal and makes it less predictable. The measurement tends to be slow as sufficient time must be allowed to allow the electrode surface to equilibrate with the tested solution.

4.3.2 Pulsed Electrochemical Detection

Pulsed Electrochemical Detection (PED) which is used almost exclusively in liquid chromatography. PED (Johnson and LaCourse, 1990) is an amperometric

detection method in which a potential waveform comprised of a sequence of pulses is imposed on the working electrode in a detector system. It is a non-stationary system. That is, solution containing analytes flows through a cell and passes by the detector. PED has been applied to numerous organic compounds following their separation by high performance liquid chromatography (HPLC). PED is best known for its superiority in detecting carbohydrates and other compounds whose complex surface electrochemistry makes their measurements using conventional, DC Amperometric Detection unreliable or insufficiently sensitive. Despite its popularity in HPLC, Pulsed Electrochemical Detection, or for that matter any other kind of Pulse Amperometric Detection, has not been used with stationary systems such as those involving electrochemical sensors.

In PED the potential is applied as a series of fast (*i.e.* about 50-400 ms) pulses of constant amplitude (FIG. 2). The current is measured at the end of the detection pulse while other pulses, of similar or higher amplitude, may be used to precondition the electrode surface either by electrochemical cleaning (*i.e.*, desorption) or reactivating (*i.e.*, regeneration of active groups on the electrode surface). The advantage of this approach is that the electrode fouling due to adsorption of the product of electrochemical reaction, or other sample components, can be greatly diminished.

4.3.3 Differential Pulse Detection

In the present invention, two new approaches, Differential Pulse Detection (DPD) and Intermittent Pulse Detection (IPD), for the measurement of current signals using disposable sensors were developed because the limitations of the preceeding detection systems did not allow detection of pathogens either with sufficient sensitivity to eliminate culturing steps or in a stationary system.

Differential Pulse Detection (DPD) involves applying a series of two pulses (FIG. 3). the first pulse is a longer "base" pulse set at the "resting" potential, *i.e.* where no significant charge transfer should be expected, or at potential that would allow measurement of a background current. The second pulse is a shorter detection pulse with

sufficient potential to electrochemically oxidize or reduce the analyte, or one of the products or reagents participating in the analyte reaction in solution phase or on the electrode surface. Current is measured at the end of both pulses and the subtracted value is used as a signal. By proper selection of the base potential, variable effects of background currents are eliminated, which is of great importance in applications employing disposable sensors.

4.3.4 Intermittent Pulse Detection

In the Intermittent Pulse Detection (IPD) a very similar approach to that used in Differential Pulse Detection is applied, but a significant difference is that instead of the "base" pulse during which controlled potential is applied to the working electrode, the working electrode is disconnected from the potentiostatic circuit of the monitor or amperometer (FIG. 4). Thus the electrode to is allowed "relax or rest," *i.e.* assume its natural potential where truly no charge transfer is occurring. The boundary conditions are restored in a natural, *i.e.* not imposed by any applied potential, way. That way, the current which is measured at the end of the detection pulse reflects the activity of the enzyme label attached to the electrode surface, and is not obscured by the accidental charge transfer process which could be the case when the base potential selected in the Differential Pulse Detection is not truly the open circuit potential. Surprisingly, the IPD greatly improves the sensitivity of the present invention. One would not expect that disconnecting the electrode would actually improve its function and the sensitivity of the assay.

Advantages of both differential and intermittent pulse measurement schemes include:

1. Electrode fouling is effectively eliminated. The measurement time, when a potential is applied to the electrode, is significantly reduced (from seconds to milliseconds) and, consequently, adsorption of reaction product(s) or other sample components is minimized, helping to maintain a steady response of the electrode.

2. The measurement is faster. Currents are measured on the millisecond time scale while the time scale of seconds or minutes is used in conventional DC Amperometric Detection. One to two seconds is sufficient to establish a "steady state" current signal and a quantifiable measurement.
3. A high rate of current measurement (about 5-50 Hz can be used, but about 10 to 25 Hz sampling rates are preferred) allows for rapid acquisition of a large numbers of data points and effectively reduced background signal interference.
4. Additional improvement of the signal-to-noise ratio can be accomplished by data averaging or FFT (fast fourier transformation) smoothing.

In measurements involving gene probes and disposable colloidal gold based sensors, the IPD is more suitable and advantageous. For example, when colloidal gold-Neutravidin (cAu/NA) sensors were used with captured biotin-HRP label, IPD produced significant enhancement of the sensitivity compared to the conventional DC Amperometric Detection. Further, after a 5 minute incubation of 1:20,000 dilution of biotin-HRP stock solution in phosphate buffered saline (PBS) and bovine serum albumin (BSA) buffer on the biosensor at room temperature, the measured currents were approximately 7-8 times higher in pulse detection compared to the conventional amperometric detection.

4.4 Probe Design and Target DNA

Short stretches (about 18-50 nucleotides) of single stranded DNA are preferred as the oligonucleotide components of the biological probes. Two oligonucleotide directed toward separate, non-overlapping regions of the target DNA sequence are used in a sandwich hybridization format. By using two non-overlapping probes to identify a target microorganism, the risk of "background noise" being interpreted as a false positive reading is reduced as compared to a system that relies on the hybridization of a single gene-probe for detection.

In certain cases, one of the probes, the capture probe, is biotinylated and is readily bound to the colloidal gold-streptavidin conjugate of the working electrode array. The other oligonucleotide probe, the detector probe, is labeled with fluorescein. A preferred detector probe comprises at least one molecule of fluorescein, a more preferred detector probe includes about two to about eight molecules of fluorescein and a particularly preferred detector probe includes about two to about four molecules of fluorescein. Anti-fluorescein antibody conjugated to an electroactive label, *e.g.* horseradish peroxidase (HRP), is used to enzyme-label the probe in certain instances.

A preferred source of target DNA is ribosomal DNA (rDNA) rather than genomic DNA because of its far greater copy number in a given cell. For example, there are approximately 1000 copies of a rDNA gene per *E. coli* cell and approximately 500 copies of an rDNA gene per eukaryotic cell. Alternatively, the source of target nucleic acid segments may be genomic DNA, RNA, cDNA or rRNA.

It is preferred that double stranded DNA be denatured before hybridization. DNA may be treated with restriction enzymes before hybridization with the probes. The stability of the hybrids and consequently the specificity of hybrid formation can be adjusted by varying the temperature and ionic strength of the solution. Other conditions that may be desirable to optimize include, but are not limited to, temperature, magnesium concentration, amount of target DNA and probes, and interference between hybridization and sensitivity.

In general, treatment with a standard lysis buffer, preferably 0.75N sodium hydroxide, is all that is required to lyse bacteria, in cultivable or uncultivable states, and release the target-DNA in a form that be detected with the claimed invention. Optimal lytic conditions may require appropriate adjustment of pH, treatment time, and possibly additional lysis and sample modifying reagents. As is common for analysis, additional modifiers may be desired to improve the performance of the system. For example, the

addition of AQ polymer improves the spreading of water samples over the surface of the test electrode, thereby improving the sensor performance.

4.5 Electrode Design

The element in the biosensor comprises a carbon electrode studded with an array of nanometer-sized, probe-coated gold particles and is capable of detecting target analyte at picogram to femtogram (10^{-12} to 10^{-15} gm) concentrations in approximately ten minutes. The assay is based on anodic stripping voltammetry at a colloidal gold test electrode and has been previously described in U.S. Patents 5,217,594; 5,368,707; 5,225,064; 5,334,296; and 5,391,272, incorporated herein by reference.

The use of high performance screen printed sensors containing an ultramicro array of colloidal gold particles is preferred in the present invention. Ultramicroelectrode arrays are more electrochemically conducting or "active" than corresponding bulk electrodes. The gold particles serve uniquely both as a high surface area immobilization support to which enzymes, antibodies or other biomolecules can be attached and as the working electrode. The ultramicro-electrode array allows enhanced sensitivity over other electrochemical approaches by having a high effective signal:noise ratio, being highly sensitive to analyte and having fast response to the analyte. A small size is also desirable under many assay conditions. The resulting system is applicable for both research and field lab applications that require monitoring large numbers of samples.

The electrical contacts are made inside the monitor with the screen-printed circuits on the plastic test strip (FIG. 5). The dark spot on the test strip is the sample well, where the unknown, *i. e.* hybridized sample, is to be placed over the working electrode. The working electrode is formed by an array of sub-micrometer size colloidal gold particles that are the immobilization support for active reactants such as the gene probes. The central crosshatched area is the test electrode (onto which colloidal gold is deposited). The test electrode is essentially surrounded by two other cross hatched areas that function as reference and counter electrodes. The three electrodes are contained

within a bean-shaped depression which serves as a sample well. Crosshatched rectangles at the right end of the strip are where electrical contact is made with the monitor.

Screen printing is the preferred method to fabricate biosensor electrodes. The electrodes are printed using a polyester screen of 240 mesh count and 36.25 angle. Appropriate conducting inks may be purchased from DuPont. Multiple overprintings of dielectric are used to build up the well to contain the sample. Colloidal gold (cAu) is deposited on the carbon working electrodes to make the cAu-modified working electrode. A silver electrode serves as the reference electrode.

The electrochemical monitor in its most basic form has a port to accept the disposable test strip, a button to begin the analysis, an LCD screen to display results. The monitor can display test results on its LCD screen or store data in internal memory. Preferably, configured with an RS-232 port, the stored data can be later uploaded to a personal computer or network for further analysis or long-term storage.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5.0 Examples

5.1 Example 1: Electrochemical Detection of Synthetic Oligonucleotide

5.1.1 Materials and Methods

5.1.1.1 Preparation of Colloidal Gold/Streptavidin (Au/SA) and Colloidal Gold/NeutrAvidin (Au/NA) Electrodes

Streptavidin solution 1 mg/ml (salt-free) was purchased from Pierce, Rockford, IL). Gold trichloride ($HAuCl_4 \cdot 3H_2O$) (Fisher Chemical Co.) was used to prepare colloidal gold sols with a particle diameter of approximately 300 Å by the method of Moremans *et al.* (1985) with the following modifications. Centrifuge gold (Au) 0.05 mg/ml stock solution, at 7,000 rpm for 50 minutes. Remove supernatant as much as possible. Collect the red pools and add distilled H_2O so that the Absorbance of the sol at 1:100 dilution in water is 0.5 at 518 nm (OD = 50). The concentration of prepared Au sol is about 2.5 mg/ml.

One mililiter of Au sol and 200 µl streptavidin solution were mixed together and 20 µl bicarbonate solution (1M $NaHCO_3$, pH 8.5) was immediately added in a glass test tube. The solution was stored in a refrigerator before use. Salt-free NeutrAvidin (Pierce, Rockford, IL) was dissolved in H_2O to make a 2 mg/ml solution. Distilled water, 500 µl, and 2 mg/ml NeutrAvidin (in H_2O), 200 µl, were mixed together and 12 µl of 1 M $NaHCO_3$, pH = 8.5 (freshly filtered) was immediately added. The solution was stored overnight in a refrigerator before use.

Gold/NeutrAvidin was mixed (1:2) with absolute ethanol (1 part Au/SA or Au/NA, 2 parts ethanol). Au/Streptavidin (Au/NA), 2.5 µl, was placed on each electrode and allowed to dry completely at room temperature. StabilCoat (50 µl, BSI) was added to each electrode and incubated for 10 minutes at room temperature. The StabilCoat was aspirated from the electrode and the sensor was allowed to dry briefly then bagged with desiccant for storage.

The current range for a base sensor is between 9 and 12 uA. Sources of minor variation between different batches of sensors may be the Au/Na sol, Au/Na strip or the

Biotin-HRP solution. Hence conditions may be optimized if desired. Variables to optimize include: 1) sensor printing parameters; 2) colloidal gold formulation and deposition; 3) polymer coating; and 4) electrode size and configuration. A relative standard deviation (RSD) of 5% or less to sensors is preferred. The SAS Institute's statistics and experimental design program (JMP®) were used to assist the optimization process.

5.1.1.2 Sample Preparation and Detection

Normally fresh or recently isolated biological samples are used for DNA isolation. Alternatively, synthetic DNA targets and human DNA from commercial sources may be used in order to optimize preferred sample conditions.

The general preferred preparation for samples suspected of containing a bacteria, such as *E. coli,* is to lyse the bacteria with sodium hydroxide, preferably with 0.75 N NaOH, for 5 minutes. A hybridization solution (2 M sodium phosphate, pH 6.5, 0.9 M NaCl, 0.12 M Tris-HCl, 6 mM EDTA, 0.002% bovine serum albumin (BSA), 0.002% polyvinylpyrolidine, 0.002% Ficoll, 0.1% pyrophosphate, or 1.05 M Tris,(pH 7.5), 0.5 mM disodium EDTA 0.05% BSA) containing detector probe, was added to the lysate and incubated at 65°C for 10 minutes. The hybridization solution was then transferred to the test strip sensor either with immobilized capture probe or NeutrAvidin/Au and incubated for 10 minutes at 65°C. The sensor was washed, with buffer equivalent to the hybridization solution without probe or target, to remove excess hybrids that did not couple to the sensor strip then 0.1 ml of HRP-conjugate, preferably HRP-anti-fluorescein antibody, was applied to the sensor and incubated at room temperature for 10 minutes. The sensor was washed and inserted into a monitoring device. After applying the substrate solution, an electrochemical signal was detected and quantitated after about 10 seconds.

5.1.1.3 Assay conditions using a model system

Target DNA, an oligonucleotide comprising 21 contiguous guanosines and 27 contiguous thymidines and designated as G21-T27, was placed in varying amounts of solution at concentrations of 0 or 3.5 nM.

When the capture probe was attached to the electrode, the detector probe, Fluorescein-A23, twenty three contiguous adenosines bonded to at least one fluorescein molecule, was added for final concentration of and 14 pM, respectively, and incubated at room temperature for 15 minutes. Test strips were then washed with PBS/0.5% BSA. Anti-Fluorescein-HRP conjugate (25 µl of a 1:1000 dilution in PBS/ 0.5% BSA from 1-2 mg/ml solution purchased from Biodesign International) was added and incubated at room temperature for 5 minutes. TMB/hydrogen peroxide substrate solution was added and the current was measured.

Alternatively, when both the capture probe and the detector probe were in the hybridization solution the following conditions were used. PBS/0.5%BSA (25 µl) containing Fluorescein-A23 and anti-Fluorescein-A23 HRP conjugate was mixed with target DNA, G21-T27 (25 µl) in PBS/0.5%BSA was mixed on the electrode and incubated at room temperature for 15 minutes. The strips were washed with PBS/0.5%BSA. TMB/hydrogen peroxide substrate solution was added and the current was measured.

5.1.2 Results

Sample volume affected assay performance as shown in Table I. Sample volumes of 100 µl or more yielded the greatest net signal indicative of the target DNA compared to the background signal produced in the absence of any target DNA.

Table I: Affect of sample volume on signal strength.

Sample Volume (μl)	Current (nA)	
	0 nM Target rDNA	3.6 nM Target rDNA
10	-167	-898
25	-165	-1241
50	-129	-1344
100	-151	-2210
1000	-120	-2199

The target DNA was detected regardless of whether the capture probe was in the hybridization solution or attached to the electrode (Table II).

Table II: Comparison of different hybridization protocols.

G21-T27 (fmol/sensor)	i/nA (1 step)	i/nA (2 step)	i/nA (3 step)
0	291	210	142
22	709	--	479
24.5	--	526	--
65	1234	--	754
74	--	1039	--
200	2572	--	2348
222	--	2811	--
600	3022	--	4577
665	--	4825	--

5.2 Example 2: Electrochemical Detection Compared to Colorimetric Detection

5.2.1 Sensitivity and quantitation

A colorimetric detection assay, Colorimetric GENE-TRAK® Assays (GENE-TRAK Systems, Framingham, MA) was compared to the present invention for sensitivity, ease of use and reproducibility.

The Colorimetric GENE-TRAK® Assays kit was used following manufacturer's procedures. In order to reduce variability in the comparison, the positive control DNA and capture and detector probes from the Colorimetric GENE-TRAK® Assays kit were also used with the present invention.

The protocol for electrochemical detection of the positive control DNA was similar to that used previously except for the following modifications. After the target-DNA and capture probe were hybridized to each other, the resulting hybrid was "captured" onto the claimed gene-probe electrode by hybridization between a poly-dA tail on the capture probe and a poly-dT oligonucleotide immobilized on the colloidal-gold electrode in place of streptavidin. The captured hybrid was incubated with an anti-fluorescein-HRP conjugate supplied by the Colorimetric GENE-TRAK® Assays kit.

Sample preparation was as follows: lysis solution (50 µl) from the Colorimetric GENE-TRAK® Assays kit was added to tubes containing 250 µl of positive or negative controls (supplied by the Colorimetric GENE-TRAK® Assays kit) and incubated at room temperature for 5 minutes. Probe solution (100 µl) from the Colorimetric GENE-TRAK® Assays kit which contained detector and capture probes was added to the tubes and incubated at 65°C for 40 minutes to allow the gene-probes to hybridize to the target DNA. Fifty microliters of the target:hybrid solution was placed onto test strips which were coated with immobilized poly-dT, and the strips were incubated at room temperature for 20 minutes to allow target:hybrids to attach to the strips. The sensor strips were washed with 1X Wash solution supplied with the Colorimetric

GENE-TRAK® Assays kit and 50 µl of Enzyme Conjugate solution as provided by the commercial kit, was added to the sample well of the test strip. Test strips were incubated at room temperature for 20 minutes then washed and 50 µl of Substrate-Chromogen solution, from the GENE-TRAK® kit, was applied per strip. Strips were inserted into a detection monitor and an electrochemical signal was measured.

Using slightly modified assay materials from the commercial kit, electrochemcial detection yielded current values of about 1053 nA and about 94 nA for the positive and negative controls respectively. In contrast, colorimetric detection yielded absorbance values of about 2.8 and about 0.13, for the positive and negative controls respectively.

5.3 Example 3: Electrochemical detection of *E. coli* and *Salmonella*

E. coli and *Salmonella* were detected using the probes and assay reagents provided by the Colorimetric GENE-TRAK® Assays kit with the streptavidin modified colloidal gold electrodes and detection monitor of Example 2 with the following modifications.

Samples containing *Salmonella*, or *E. coli* or neither (negative controls) were incubated with a 50:50 mixture of the *E. coli* and *Salmonella* detection probes from GENE-TRAK for 15 minutes at 65°C. Aliquots of 100 µl of the samples were then transferred to electrodes and incubated at room temperature for 10 minutes. The electrodes were washed with PBS, 0.5% Tween-20. Fifty microliters of anti-fluorescein-HRP solution was placed on the electrodes and incubated for 5 minutes at room temperature. The electrodes were washed with PBS, 0.5% Tween-20. Current was measured using TMB/Peroxide solution and the detection monitor. Electrochemical detection of the bacteria are shown in Table III.

Table III: Electrochemical detection of *E. coli* and *Salmonella*

Sample	Current (nA)
Negative Control*	-375
Negative Control*	-401
E. coli Positive Control**	-1307
E. coli Positive Control**	-1007
Salmonella Positive Control**	-1731
Salmonella Positive Control**	-1655

*A mixture of Negative control solutions provided with the Colorimetric GENE-TRAK® *E. coli* and *Salmonella* Assays which contain killed bacteria.

**Synthetic oligonucleotides provided with the with the Colorimetric GENE-TRAK® *E. coli* and *Salmonella* Assays and diluted 1:3 with bovine serum albumin in aqueous solution.

5.4 Example 4: Sensitivity of Detection and Quantitation of *E. coli*

To determine the lower limit of sensitivity, two approaches were used. In the first approach, the capture probe was pre-immobilized on the electrode biosensor through NeutrAvidin binding. In the second approach, the capture probe, examined at a fixed concentration of 200 µM, was allowed to hybridize to *E. coli* rDNA in solution, along with the detection probe. The hybrid was captured at the electrode surface through biotin/NeutrAvidin binding. In both cases, the concentration profile of the detector probe was examined using the procedure described under Section 5.4.1 Material and Methods.

5.4.1 Optimization of probe concentration and hybridization

5.4.1.1 Materials and Methods

E.coli cells were lysed with 0.75N sodium hydroxide for 5 minutes. A hybridization solution containing detector probe was then added to the lysate and incubated for 10 minutes at 65°C. The hybrid solution was then transferred to the test strip which either included immobilized capture probe or NeutrAvidin/Au and incubated for 10 minutes at 65°C. The sensor strip was then washed to remove unbound hybrid.

HRP-anti-fluorescein, 0.1 ml, was applied to the sensor and incubated for 10 minutes at room temperature. The sensor was again washed and then inserted into a detection monitor. The substrate solution (0.001% hydrogen peroxide) was applied and after about 10 seconds the electrochemical signal had been measured by the monitor.

A capture probe was prepared which was biotinylated at the five prime end and contained the DNA sequence 5'-TCAATGAGCAAAGGTATTAACTTTACTCCCTTCCT-3' (SEQ ID NO:1). The detector probe contained the DNA sequence 5'-TGAAAGTACTTTACAACCCGAAGGCCTTCTTCATA-3' (SEQ ID NO:2), and a single fluorescein (FL) group was attached at the 5' and 3' ends, respectively, such that the probe was labeled with two FL groups.

5.4.1.2 Results

In the first approach using the immobilized capture probe, increasing the detector probe concentration increased the assay signal. The signal peaked at about 30 nM of detector probe in the hybridization solution. Higher concentrations of detector probe yielded lower signal-to-noise ratios. Data are given in Table IV.

Table IV: Detection of *E. coli* (in mA) using immobilized capture probe

E. coli	Detector probe			
10^6 cells/ml	16 nM	31 nM	65 nM	125 nM
0	0.032	0.028	0.031	0.028
0.44	0.038	0.045	0.037	0.045
1.46	0.055	0.08	0.054	0.08
4.4	0.092	0.175	0.082	0.175

In contrast, the second approach where the capture probe was included in the hybridization solution yielded greater sensitivity of detection (Table V). The highest sensitivity was achieved at a detector probe concentration of 100 nM in the hybridization

solution. At higher concentrations of detector probe, the signal-to-noise ratio decreased. Based on these results, the preferred conditions for the assay format are 200 nM of capture probe and 100 nM of detector probe in the hybridization solution. This latter format reliably detected 0.5 million cells/ml of sample; whereas, the former approach only detected 2 million cell/ml of sample.

Table V: Detection of *E. coli* using soluabilized capture probe

E. coli	Detector probe		
10^6 cells/ml	25 nM	50 nM	100 nM
0	0.043	0.048	0.05
0.47	0.075	0.085	0.098
4.7	0.398	0.418	0.52
47	2.7	2.93	3.44

5.4.2. Alternative probes

To determine if altering the number of fluorescein groups available changed the sensitivity of the assay, detection probes that had different numbers of FL groups attached to SEQ ID NO:2 or that had altered attachments of the FL groups to SEQ ID NO:2 were prepared. These detection probes are summarized in Table VI.

Table VI: Generalized detection probe structures.

Number of FL molecules	Probe structure
1	5'-SEQ ID NO:2-FL
2	5'-FL- SEQ ID NO:2-FL
4	5'FL-T-FL- SEQ ID NO:2-FL-T-FL
8	5'-FL-FL-FL-FL- SEQ ID NO:2-FL-FL-FL-FL

In addition, a new probe set which is complementary to different regions of rDNA of *E. coli*, was designed to determine if the specificity detection was influenced by the region

of the DNA to which the probes hybridized. The capture probe of the second probe set comprised theDNA sequence 5'-GTCTCACGGTTCCCGAAGGCACATT-3' (SEQ ID NO:3) and was biotinylated at the 5' end. The detector probe comprised the DNA sequence

5'-TCTCTGAAAACTTCCGTGGATGTCA-3' (SEQ ID NO:4) and included two molecules of fluorescein dUTP, one attached at each of the respective ends.

The assay conditions and procedure were the same as outlined above using the second approach in Section 5.4.1.1 in which the capture probe, 200 nM, and detector probe, 100 nM, were present in the hybridization solution. The *E. coli* sample solution concentration was 30 million cells/ml.

5.4.2.1 Results

As shown in FIG. 6, the signal strength increased proportionally as a function of the number of fluorescein moieties present for probes labeled with either one, two or four fluoresceins. The presence of eight fluoresceins did not enhance signal strength possibly due to steric hindrance of the anti-fluorescein HRP conjugate binding to the fluoresceins. The results also indicate that spacing the fluoresceins apart with one thymidyl residue significantly reduced the steric hindrance. With the four fluorescein labeled probe, the sensitivity of detection of *E. coli* was improved to 200,000 cells/ml.

Detection with the second set of probes, utilizing the oligonucleotides shown in SEQ ID NO: 3 and SEQ ID NO:4, was also successful. Results showed increased specificity and sensitivity compared to the first probe set, represented by SEQ ID NO:1 and SEQ ID NO:2. With the first probe set the lower limit to sensitivity was about 500,000 cells/ml; whereas the lower limit of detection with the second probe set was about 50,000-100,000 cells/ml. These results demonstrate that the selection of the regions to which the probes hybridize, the assay conditions used, and the concentrations of the probes all affect the sensitivity of detection.

Based on these results, the detection limit of the electrochemical detection is about 0.01 nM, or 50 attomoles/sensor. The lowest noise level that was observed with the Au/NA sensors for HRP detection was about 0.02-0.04 uA, of which about 0.02 uA is present in the substrate solution alone. Currents were observed to lose linearity above aboutr 10 uA. Thus the preferred current detection range for the quantitation of a microbial target is between about 0 uA and about 10 uA.

5.5 EXAMPLE 5: *E. coli* Detection in Meat Extract

In this example it was determined that a food sample did not cause matrix interferences in the electrochemical determination of *E. coli*.

5.5.1 Materials and methods

Approximately 10 grams of beef was cut into approximately 50 pieces (about 200 mg per piece). 40 ml of Butterfields Buffer was added to the meat and shaken for 1 minute. The meat was allowed to settle and the "Meat Extract" was removed and used as described below.

A stock solution of *E. coli* was diluted 1:10,000; 1:100,000; and 1:1,000,000 and added to the Meat Extract (Meat Extract without *E. coli* acted as a negative control.) One ml aliquots were placed on 3M Petrifilm Total Coliform/*E. coli* plates (3M, Minneapolis, MN) (a 1:10 dilution of the 1:100,000 sample and a 1:100 dilution of the 1:10,000 sample was used), and 1.2 ml each of these samples were added to a 1.5 mL microfuge tube containing 26 mg of Lennox L Broth (1 g Casein Enzymatic Hydrolysate: 0.5 g Yeast Extract: 0.5 g NaCl: 100 mg Glucose). The tubes were shaken and vortexed until the powder was completely dissolved, then incubated at 37°C for 3 hours. Microfuge tubes were spun at 14,000 rpm for 3 minutes and supernatant was removed with a transfer pipet. Cells were resuspended in 75 µl of 0,125 N NaOH and incubated at room temperature for 5 minutes. Probe/Neutralization Buffer (25 µl) (2 M Phosphate/0.9 M NaCl/6 mM EDTA/0.1% SDS) containing 200 nM Probe #39 and 100 nM Probe #23 was added to each tube and incubated at 65°C for 10 minutes. An aliquot (100 µl) of sample was applied to Au/NeutrAvidin electrodes and incubated at 65°C for 10 minutes. The

electrode was washed with *E. coli* Wash Buffer (GeneTrak, Framingham, MA) then incubated with 50 µl of 1:200 anti-Fluorescin-HRP in PBS/Casein at room temperature for 10 minutes and again washed with *E. coli* Wash Buffer (GeneTrak, Framingham, MA). Fifty microliters of K-Blue Substrate (Elisa Technologies) was added to the sample/electrode and the current was measured after 10 seconds. The results are shown in Table VII below.

Table VII: Detection of *E. coli* in Meat Extract

Sample Dilution (*E. coli*)	CFU/ml (24 hour Petrifilm)	Electrochemical Current (-nA)	
Negative control	0	313	238
1:1,000,000	200	759	668
1:100,000	1,900	2596	2596
1:10,000	20,000	OFFSCALE	OFFSCALE

5.6 EXAMPLE 6: Gene-Probes to Detect Enterically-Transmitted RNA Viruses

This example describes a rapid detection system for enterically-transmitted virus pathogens. Polio virus is used as the exemplary RNA virus.

Hep-2 (human larynx epidermoid carcinoma) and Vero (African green monkey kidney) cells were grown in culture and infected with polio virus (Sabin Type I, LSc 2ab). Types 2 and 3 polio virus can also be used. From these cultures 1.6 liters of solution containing polio virus was isolated and stored in frozen aliquots. The number of plaque-forming units/ml in the isolated virus was obtained ($10^{-6.5}$ pfu/ml). The virus RNA was then purified using a viral RNA prep kit (Qiagen Corporation) and this vRNA was used in electrochemical experiments.

The cDNA for Type I polio virus obtained from ATCC was cloned in competent *E. coli*, isolated, and used in preparation of several detector probes. Detector probes with

multiple digoxigenin (DIG) labels were synthesized by PCR™ using this cDNA, a DIG-labeling kit (Boehringer-Mannheim, Indianapolis, IN), and three sets of primers synthesized by National Biosciences, Inc. Two of the three probes (LPD2 and LPD3) were the same as those used by Bosch *et al.* (1996) for detection of polio virus at 5×10^3 particles/ml without target amplification. The first probe (LPD1) was generated in a different manner from Bosch *et al.* (1996) by use of PCR™ labeling with DIG and was complementary to bases 651-2099 of the polio virus. Another primer (LPD4) complements bases 700-2099 of the polio virus.

Synthesis with multi-DIG labeling was successful for two of the three detector probes. Dot blot tests indicated that the probe complementary to bases 3401-4686 of the virus was most heavily labeled with DIG, and this probe was used to detect purified polio virus, RNA transcripts, and cDNA for the virus on Au/NeutrAvidin electrodes in a system that incorporated a 5′-biotinylated capture probe with a sequence complementary to bases 601-625 of Type I polio virus. This capture probe is nonspecific and is useful in the detection of many types of enteroviruses (Nottay *et al.* 1995).

Results indicated that the DIG labeled probes can be employed to electrochemically detect 100 pM of polio cDNA without optimization of the system. Electrochemical response to the target polio cDNA is high. But there is also increased background signal from the multiply labeled DIG probes and possible competition from the negative strand of cDNA binding a portion of the probes.

All of the compositions, methods and apparatus disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions, methods and apparatus and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described

herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

6.0 References

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCAATGAGCA AAGGTATTAA CTTTACTCCC TTCCT 35

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
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TGAAAGTACT TTACAACCCG AAGGCCTTCT TCATA 35

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(A) LENGTH: 25 base pairs
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GTCTCACGGT TCCCGAAGGC ACATT 25

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(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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TCTCTGAAAA CTTCCGTGGA TGTCA 25